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Mechanisms of depolymerization and activation of a polysialic acid and its tetramer by hydrogen peroxide

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ABSTRACT

Naturally occurring polysialic acid (PSA), appropriately functionalized, has been widely used in different biological products. The present paper describes an original approach which enables to both activate and depolymerize the PSA, by reacting with hydrogen peroxide. In order to understand the mechanisms, we investigate the course of H_2O_2 reactions with the native PSA and with a simpler model, the tetrasialic acid (4SA). Three recurrent reactions were observed. First, we detected a very fast and irreversible decarbonylation at the reducing end of the polysaccharide. Then, the hydroxyl radicals (generated via the Fenton reaction) were responsible for the depolymerization of glycosidic linkages by substitution reactions. Finally, the oxidation of hydroxyl groups led to the formation of carbonyl groups and the carbohydrate's activation.

1. Introduction

\Polysialic acids (PSA) are linear polymers of sialic acid (SA), a nine-carbon carboxylated alpha-keto sugar. They occur in nature, primarily as carbohydrate chains of bacterial polysaccharides like *Escherichia coli* K_{235} (Barry, 1958). In animals, they play an important role in modulating neuronal development as a component of the neural cell adhesion molecules (Finne, Finne, Deagostini-Bazin, & Goridis, 1983).

A large structural variability offered at the monomer level (acetyl, methyl, phosphate or sulfate groups) can be seen at the polymer level in vertebrates' tissues. But in bacteria, PSA are only observed as unbranched polymers composed of repeating units of α-(2→8), α-(2→9), or alternating α-(2→9)/α-(2→8) sialic acid residues, partially *O*-acetylated. Their structures (Fig. 1) have been studied in detail in the literature (Lemercinier & Jones, 1996; Lifely, Nowicka, & Moreno, 1986; Mühlenhoff, Eckhardt, & Gerardy-Schahn, 1998).

Because of their presence at the surface of numerous cells, they have been used in a wide range of biomedical applications after some chemical modification (Gregoriadis, Jain, Papaioannou, & Laing, 2005; Lindberg, 1999).

Some activation chemistries might lead to depolymerization of the polysaccharide while some will not lead to deliberate degradation. The most used technique to functionalize the PSA is to introduce carbonyl groups by periodate-mediated oxidation of the adjacent hydroxyls at C-7, C-8, and C-9 (Gregoriadis et al., 2005). Other chemistries involve the generation of amine groups by deamidification of the N-acetyl group (Cuello et al., 2007), the cyanylation of hydroxyl groups creating cyanoesters or the reductive amination with ammonium acetate of the ketone at the reducing end (Bröker, Dull, Rappuoli, & Costantino, 2009).

A less common technique, using hydrogen peroxide (H_2O_2), has also been developed (Ryall, 2003). The treatment with H_2O_2 is meant to serve two purposes. First, the combination of heat and H_2O_2 will lead to the depolymerization of the PSA. Second, the chemical nature of the reaction will activate the PSA through the introduction of carbonyl groups. The efficiency of this kind of reactions has already been demonstrated with other polysaccharides (Klein-Koerkamp et al., 2009), however, the exact mechanism has still to be resolved.

Accordingly, an investigation into the complex reaction mechanism between H_2O_2 and PSA was performed using the monomer subunit, *N*-acetyl neuraminic acid (SA) as a model (Neyra et al., 2014). It was demonstrated that, after an irreversible decarbonylation reaction at the reducing end, the SA was oxidized and fragmented into a mix of carboxylic acids and carbonyls via a radical mechanism and heterolytic alpha-hydroxy-hydroperoxide cleavages (αHHP).

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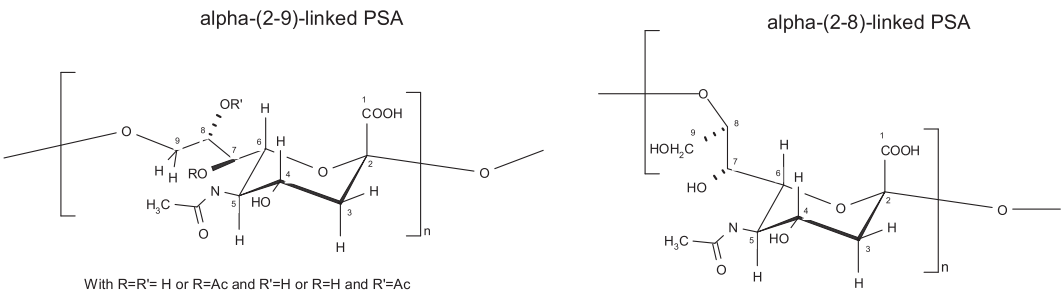


Fig. 1. Structure of α-(2 → 9) and α-(2 → 8) linked PSA.

Similar studies were initiated to elucidate the mechanism of H_2O_2 treatment on a polysaccharide, α-(2 → 9)-linked PSA, using two approaches (Fig. 2). The products obtained after the depolymerization process (for either approach) were characterized by HILIC-MS (hydrophilic interaction chromatography coupled to mass spectrometry), NMR, and HPSEC (size exclusion chromatography). For the first experiment, the PSA was depolymerized by H_2O_2 oxidation. Then, the glycosidic bonds of the depolymerized PSA (dePSA) easily cleaved in a mild acidic conditions (Jennings & Bhattacharjee, 1977; Manzi, Higa, Diaz, & Varki, 1994), were hydrolyzed and the SA residues (oxidized or not) were analyzed by the LC-MS method. Conversely, in the second set of experiments, the PSA was hydrolyzed under mild acidic conditions to the tetra sialic acid (4SA) detected by ESI-M. Then, the 4SA, purified by anion exchange chromatography, was depolymerized and oxidized by H_2O_2. Using either depolymerization approach, the resulting SA residues were characterized and compared.

2. Experimental procedures

2.1. Reagents

α-(2 → 9)-linked PSA was supplied by a commercial manufacturing group. All other reagent grade chemicals were purchased from Sigma-Aldrich. The solutions were prepared with Millipore-quality water (Milli-Q plus, Ultrapure water system, 18 MΩ cm).

2.2. Oxidations by hydrogen peroxide

2.2.1. First set of experiments

α-(2 → 9)-linked PSA was dissolved in sodium acetate (50 mM, pH 6) and hydrogen peroxide was added to a final concentration of 1% (w/v). The sample was stirred at 70 °C and depolymerized. The depolymerized PSA (dePSA) was dialyzed three times against water (MWCO 3.5 kDa membrane, Spectra/Por® 7) and lyophilized.

Then, the glycosidic bonds of dePSA were hydrolyzed by heating at 70 °C, pH 4 (in acid acetic) for about 6 h. The hydrolyzed samples were cooled to room temperature, neutralized to pH 6, and analyzed by HILIC-MS.

2.2.2. Second set of experiments

α-(2 → 9)-linked PSA, dissolved in acid acetic pH 4, was hydrolyzed at 70 °C during 10 h and dialysis against water (MWCO 1 kDa membrane, Spectra/Por® 7). The HPSEC and HILIC-MS analyses of the desalted solution revealed the presence of several species, among them the tetrasialic acid, 4SA.

For the isolation of 4SA, an approach similar than the one described by Costantino et al. (1999) was used. An ÄKTA™ pure chromatographic system was equipped with an anion exchange column (HiPrep™ QFast Flow). The hydrolysate diluted in equilibrating buffer (MilliQ water) was applied onto the column and the column was washed with 5 column volumes (CV) of water. The oligosialic acids were eluted by linear gradient elution with 0%–40% elution buffer (up to 200 mM NaCl) in 40 CV. Eight different species were isolated, desalted, lyophilized, and analyzed by electrospray mass spectrometry. The molecular weight of one of the species was consistent with the tetrasialic acid structure.

After characterization, the purified 4SA (20 mg) redissolved in 16 mL of sodium acetate (50 mM, pH 6) was treated with a large excess of H_2O_2 during 5 h at 70 °C. To confirm a radical mechanism and the presence of hydroxyl radicals observed during our prior studies, two other reactions were carried out in the same experimental conditions in presence of either EDTA (2.5 μg/mL) or 25 mM of 3,3,5,5-tetramethyl-1-pyrroline-1-oxide (TMPO), a free radical trap. The oxidized products were dialyzed three times against water (MWCO 0.1 kDa membrane, Spectra/Por® CE), lyophilized, and analyzed by HILIC-MS.

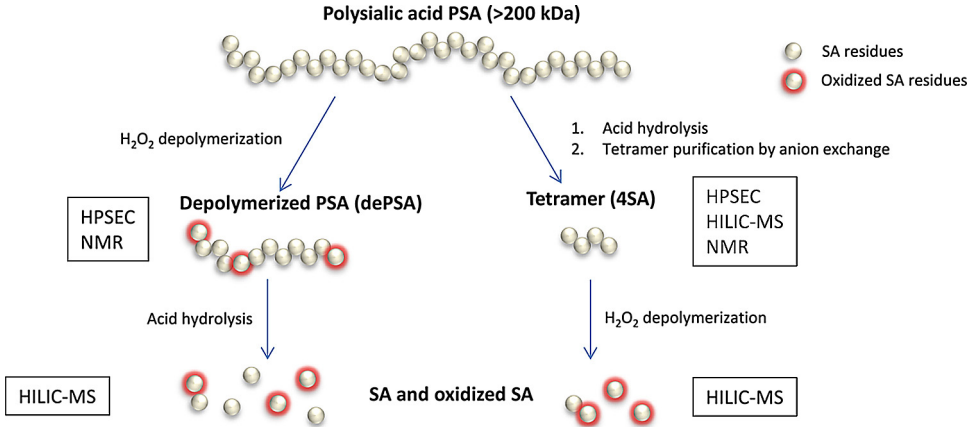


Fig. 2. Strategy followed to characterize the H_2O_2 degradation products of polysialic acids.

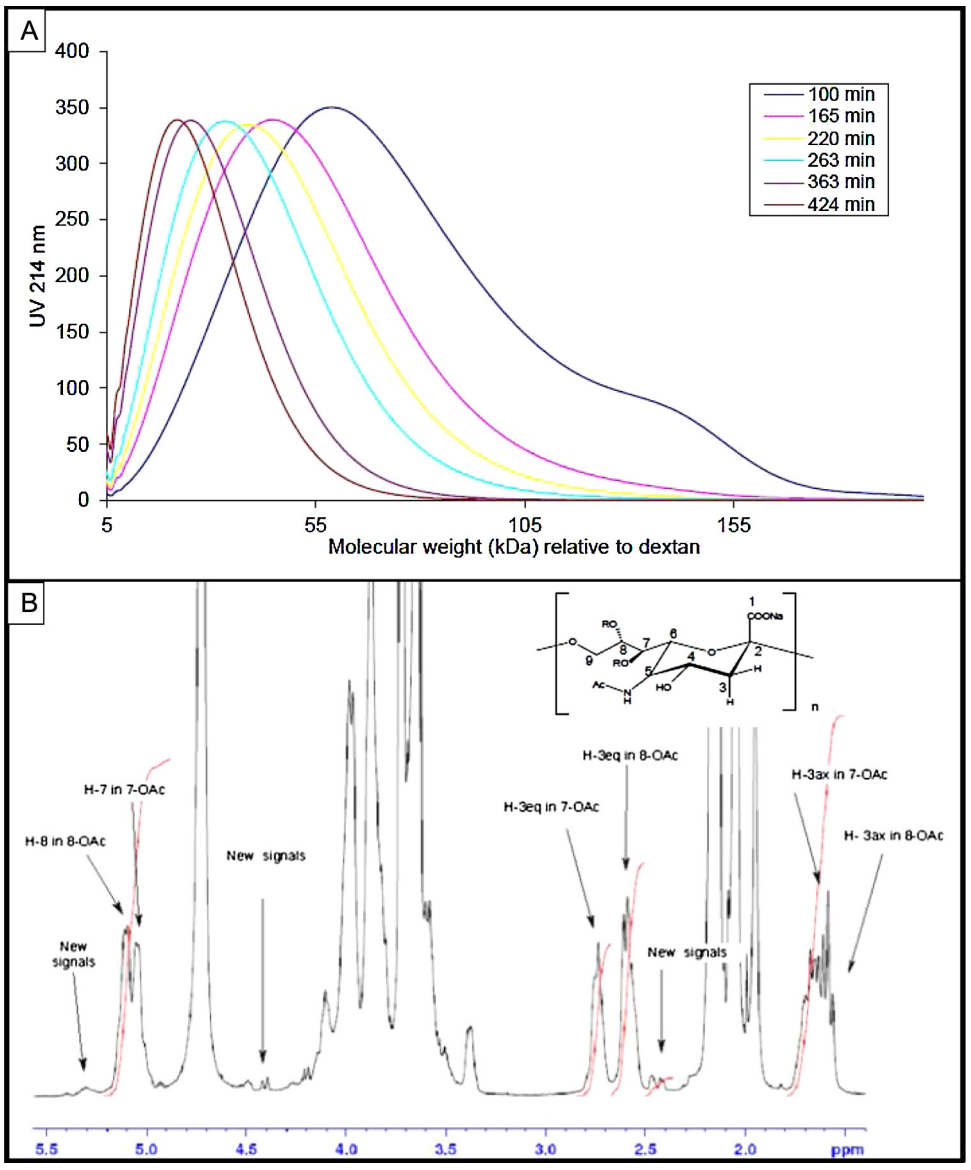


Fig. 3. (A) HPSEC analysis during the PSA depolymerization and (B) ^{1}H NMR spectrum at 500 MHz of the H_2O_2 depolymerized polysaccharides (dePSA).

2.3. Analyses

2.3.1. HPSEC analyses

Size exclusion chromatography during the H_2O_2 depolymerization and acid hydrolysis was conducted using two PL aquagel OH-30 (Agilent) columns in series, after a PL aquagel OH column guard. 50 μL sample was eluted with phosphate buffered physiological saline (0.01 M, pH 7.1) at a flow rate of 0.8 mL/min and analyzed on an Agilent 1200 series system composed of a UV (214 nm) and refractive index detectors. The chromatograms were recorded and analyzed with the Agilent Chemstation software. Dextran polysaccharides (commercially available) from different molecular weights were used as standards for the calibration curve.

2.3.2. HILIC-MS analyses

Oxidized and derivatized products were analyzed using HILIC (hydrophilic interaction liquid chromatography) coupled to ESI-MS (electrospray ionization mass spectrometry).

Samples were brought to an acetonitrile content of 80%. LC-MS analyses were performed on an Agilent 1200 LC/MSD system equipped with a UV detector (at 214 nm). A Luna® HILIC diol column (length 150 mm, diameter 4.6 mm, particles 3 μm, Phenomenex) was used. 10 μL sample was injected to the column. Tetramers were separated with acetonitrile:ammonium formate (70:30, v/v, pH 6), while monomer fragments were separated with acetonitrile:ammonium formate (80:20, v/v, pH 6) at a flow rate of 0.8 mL/min, during 25 min. The column temperature was maintained at 30 °C. The electrospray source was set in negative ionization mode with fragmentor voltage 50 V, capillary voltage 3000 V, drying gas temperature 350 °C, and scan range from 80 to 2000 Da. The control of the chromatographic system and data acquisition was achieved with the software Agilent Chemstation.

2.3.3. NMR measurement

To follow the oxidation of PSA during the reaction, samples were regularly taken from the solution, dialyzed against water to remove the salts and the excess of H_2O_2, lyophilized, and redissolved in D_2O.

^{1}H NMR spectra were recorded at 30 °C with a Bruker Avance II spectrometer at 500 MHz equipped with a cryoprobe. The data

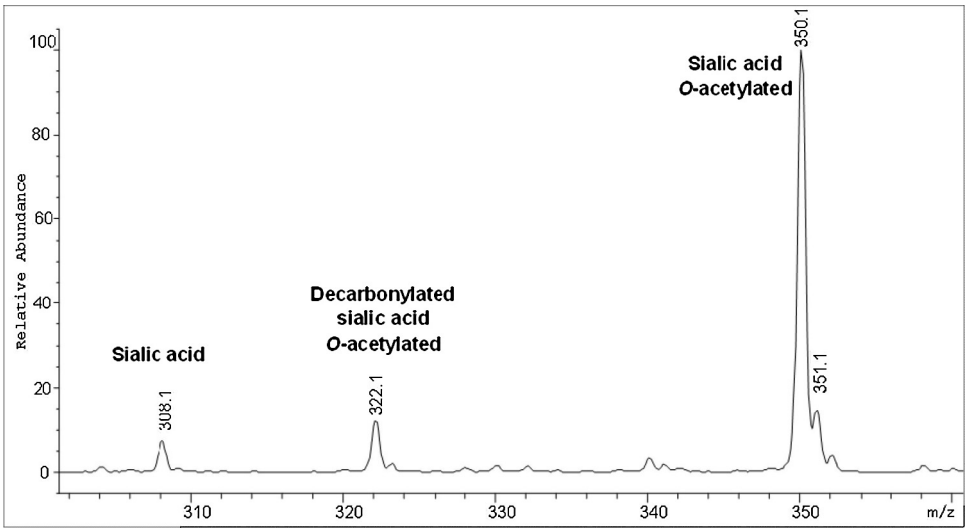


Fig. 4. Mass spectrum of the acid-hydrolyzed dePSA.

were recorded using the Bruker TopSpin™ software. The spectra were referenced with respect to an internal standard TSP-d_4 (sodium tetra deuterated trimethylsilyl propionate), at 0 ppm (^{1}H).

3. Results and discussion

The action of the hydrogen peroxide depolymerization of polysaccharides has been thought to involve the highly reactive hydroxyl radical HO• (Higashi et al., 2012; Wu, Xu, Zhao, Kang, & Ding, 2010) generated by metal ions-catalyzed decomposition of H_2O_2 (Fenton reaction) or by irradiation under ultraviolet light (homolytic fission). The mechanism of the HO• attack appears to be very complex and non-specific leading to formation of carboxylic acid and carbonyl moieties when studied using sialic acid monosaccharide (Neyra et al., 2014). The aim of this work was to propose a possible mechanism for the degradation and activation of PSA by H_2O_2.

3.1. First set of experiments

Previously (Neyra et al., 2014), we demonstrated that the metal ions present as traces in the reaction vessel were enough to generate hydroxyl radicals via the Fenton reaction. In the present work, these free radicals were capable of randomly attacking the PSA resulting in its size reduction. The molecular weight of the samples, during the depolymerization reaction, was determined by HPLC, as described in Section 2.3.1 and dePSA with an average of 30 repeating units was obtained (Fig. 3A). Additionally the results show that the size distribution decreased as the MW decrease, suggesting that the larger PSA were preferentially fragmented than the low MW polysaccharides.

The ^{1}H NMR spectrum at 500 MHz of the H_2O_2 depolymerized polysaccharides (dePSA) is shown in Fig. 3B. It presents the same chemical shift values and signal intensity described by Lemercinier and Jones (1996) for the native polysaccharide (see Supplementary data) confirming that the polysaccharide structure inside the chains remains unchanged following hydrogen peroxide treatment. The degree of *O*-acetylation stays the same (around 90%). The only detectable difference between this spectrum and the one obtained for the native polysaccharide is the presence of low-intensity signals around 5.3, 4.4, 2.5 ppm, which could be assigned to the aldehydes (hydrate form) introduced by oxidation with H_2O_2 (peak at 5.3 ppm) and to some new decarbonylated extremities of the dePSA. Indeed, the peaks at 2.5 and 4.4 ppm have similar chemical shifts than the ones observed previously (Neyra et al., 2014) for the protons H-2a, H-2b, and H-3 of the decarbonylated SA (see Supplementary data).

Further confirmation of structural integrity for internal sialic acid repeat units was provided by LC-MS. The depolymerized PS was hydrolyzed by acid treatment. The resulting fragments were then analyzed and showed intact sialic acid.

The mass spectrum of the acid hydrolyzed sample shows a major peak at 350 Da $[SA\text{-}H]^-$ corresponding to the intact sialic acid, *O*-acetylated (Fig. 4). Two other peaks with a lower intensity are also detected. The first one is assigned the de-*O*-acetylated SA, at 308 m/z ($[M\text{-}H]^-$). The second one, with a mass of 322 Da $[M\text{-}H]^-$, is attributed to the DSA. This structure, characterized by Iijima, Takahashi, Namme, Ikegami, and Yamazaki (2004) and Iijima, Takahashi, Ikegami, and Yamazaki (2007) and in our previous study between the monomer SA and H_2O_2 (Neyra et al., 2014), could be a result from peroxide based cleavage.

Other peaks are observed but their intensities are too small to assign them (Signal-to-Noise ratio too difficult to distinguish). Analysis of the composition of the H_2O_2 depolymerized tetrasialic acid may give additional data and help us to identify these compounds.

3.2. Second set of experiments

The first set of experiments confirmed the structural integrity of internal sialic acid residues after hydrogen peroxide based oxidation. However, characterization of the terminal ends of depolymerized polysaccharide proved to be difficult due the disparity in signal intensity with the internal sialic acid residues. Accordingly, tetrasialic acid was used as a model to understand the effects of peroxide based oxidation on the terminal ends of the polysaccharide.

First, partial acid hydrolysis of the glycosidic bond of the PSA led to several species, visible by HPSEC. Preparative ion exchange chromatography, conducted as described in Section 2.2.2, successfully resolved oligosialic acid into more than 8 different peaks (Fig. 5A–H). The HPSEC chromatogram of each fraction (Fig. 5B) shows a high efficiency of this purification.

Mass spectrometry permitted the correlation between the peak in the chromatogram and the degree of polymerization (ranging from 1 to 8). Fig. 6 shows the mass spectrum and the peaks

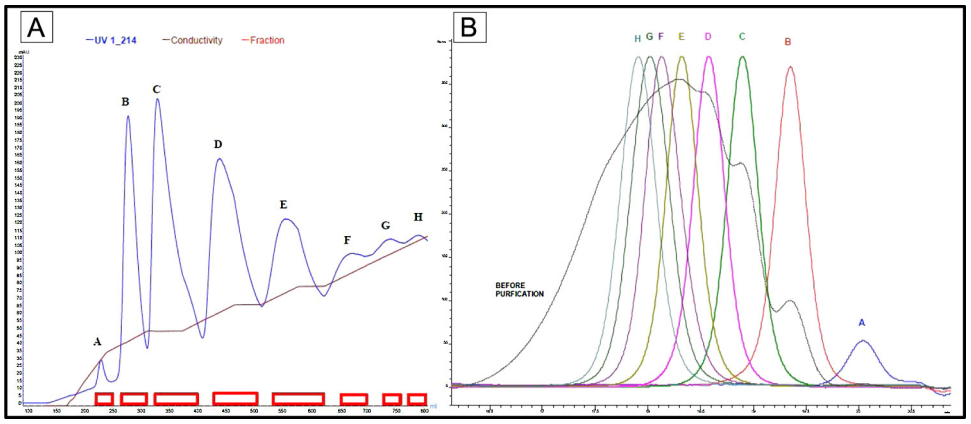
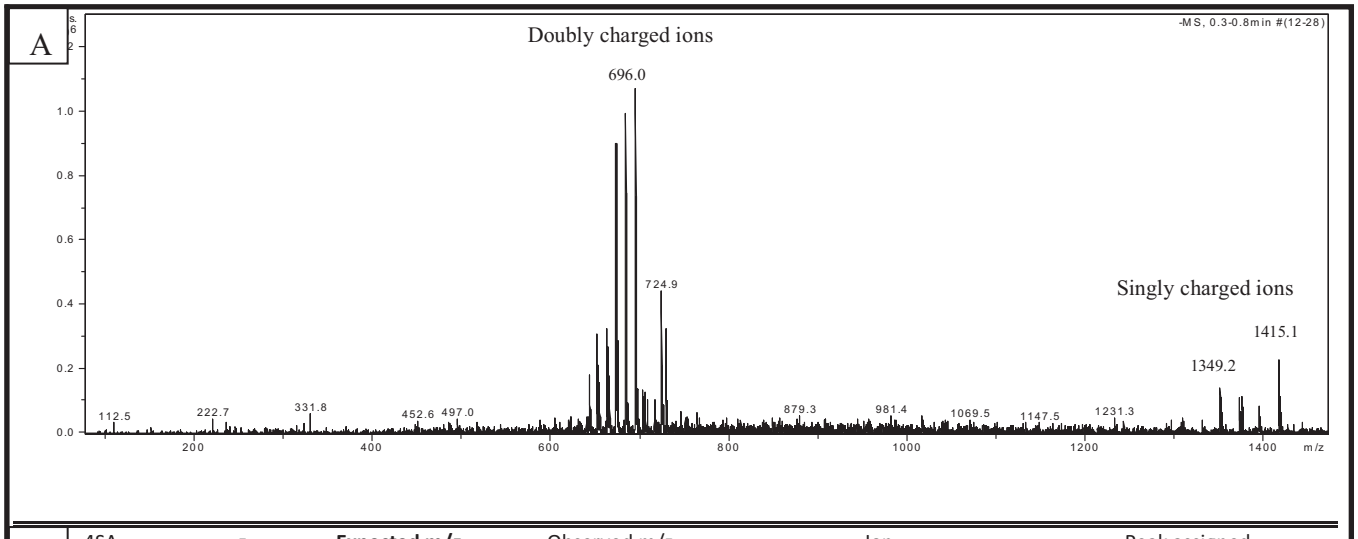


Fig. 5. Separation of the oligosialic acids by anion exchange chromatography (A) and HPSEC chromatogram at 214 nm of each fraction (B).

assignment of the fraction D, consistent with the tetramer structure. The major ion observed corresponds to the four times deprotonated molecular ion, with two sodium counter ions attached. Minor species are attributed to the loss of acetyl group, water or sodium ions form the major ion.

The purified 4SA was then oxidized by a H_2O_2 in large excess. Three successive reactions were observed by mass spectrometry. (1) After 18 min reaction, no more 4SA was detected by HILIC-MS. As noticed with sialic acid and PSA, the reducing end of 4SA was decarbonylated and the 3SA-DSA was formed. (2) Then, we observed a depolymerization reaction at the glycosidic bond followed by the quick decarbonylation reaction of the depolymerized structures. No intact 3SA, 2-SA or SA was detected but only 2SA-DSA, SA-DSA, and DSA. (3) The last kind of reaction observed was the formation of oxidation products. A plausible reaction pathway previously described (Neyra et al., 2014) would involve a non-selective hydrogen abstraction on the glycerol chain of SA residues (C-7, C-8, or C-9), by a radical hydroxyl HO•. The radical



B

4SA	z	Expected m/z	Observed m/z	Ion	Peak assigned
	1 charge	1349	1349.2	$[4SA+4Ace-H]^-$	4SA + 4Ace
			1371.2	$[4SA+4Ace+1Na-2H]^-$	4SA + 4Ace + Na
			1393.2	$[4SA+4Ace+2Na-3H]^-$	4SA + 4Ace + 2Na
			1415.1	$[4SA+4Ace+3Na-4H]^-$	4SA + 4Ace + 3Na
	2 charges	674	644	$[4SA+3Ace-H2O-2H]^{2-}$	4SA + 3Ace - H_2O
			653	$[4SA+3Ace-2H]^{2-}$	4SA + 3Ace
			664	$[4SA+3Ace+1Na-3H]^{2-}$	4SA + 3Ace + 1Na
			674	$[4SA+4Ace-2H]^{2-}$	4SA + 4Ace
			685	$[4SA+4Ace+1Na-3H]^{2-}$	4SA + 4Ace + 1Na - 3H
			696	$[4SA+4Ace+2Na-4H]^{2-}$	4SA + 4Ace +2Na - 4H

Fig. 6. Mass spectrum (A) and peaks assignment (B) of the purified tetrasialic acid.

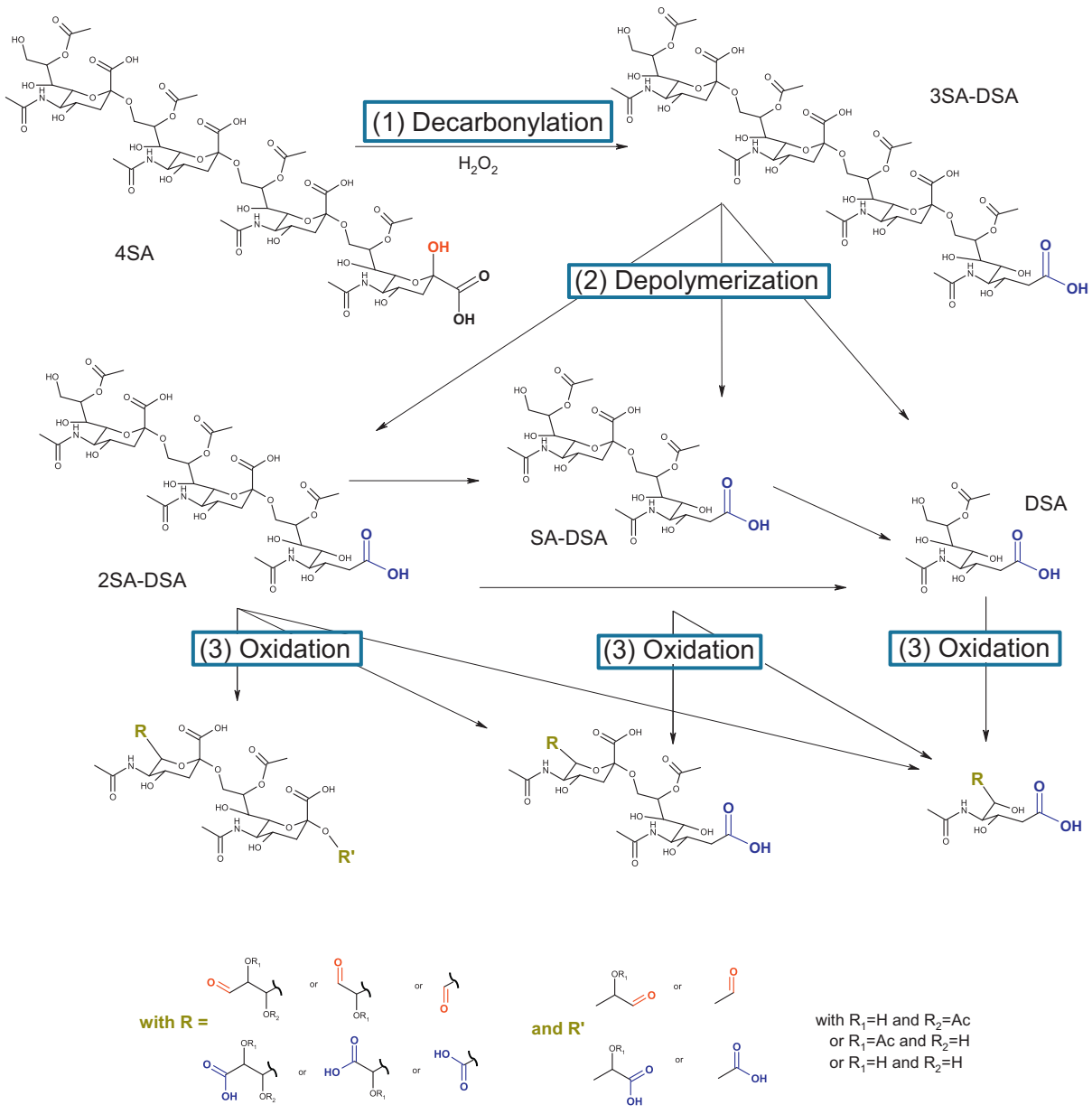


Fig. 7. H_2O_2 degradation pathway of tetrasialic acid 4SA.

intermediate formed would then react with H_2O_2 affording a carbonyl group. To finish, a nucleophilic action of H_2O_2 to the carbonyl group, followed by a heterolytic alpha-hydroxy-hydroperoxide cleavage (α-HHP) cleavage of the glycerol chain would lead to the formation of carboxylic acids and aldehydes and eventually a depolymerization (if the cleavage occurred intra-chain).

After 5 h reaction, only DSA (*O*-acetylated or not) and oxidized DSA residues were detected by HILIC-MS. The possible degradation pathway of 4SA is proposed in Fig. 7.

The experiment with 4SA was repeated twice, in presence of 2.5 ppm EDTA (a metal chelator) and 25 mM TMPO (a free radical trap) to examine the degradation mechanism. For both reactions, the kinetics of the decarbonylation reaction and formation of 3SA-DSA was the same than in regular conditions. This result confirmed that the decarbonylation reaction is not related to the presence of free radical. But then, in presence of EDTA, almost no depolymerization was detected and after 5 h reaction, 3SA-DSA remained as the main product. This proved that when traces of metal ions present in the solution were chelated by EDTA, the reaction kinetics severely decreased. However, it did not stop it completely as the EDTA-metal ion complexes were still able to participate in the Fenton reaction (Li et al., 2007). TMPO had a similar impact than EDTA. The reaction rate was clearly inhibited due to the fact that a part of the hydroxyl radicals were trapped by the free radical scavenger and a majority of 3SA-DSA was still intact at the end of the experiment.

The experiments with free radical trap and metal chelator confirmed that the depolymerization step of PSA involved a free radical reaction. The proposed mechanism of depolymerization is depicted in Fig. 8. The first step is a substitution reaction at the anomeric carbon between a hydroxyl radical and the adjacent sialic acid residue. The newly formed sialic acid radical can then abstract a hydrogen atom from H_2O_2 or another hydrogen donor. This mechanism supports the notion that hydroxyl radicals can simply cleave any randomly encountered glycosidic linkage in PSA. Almost instantly after the glycosidic bond cleavage, the new reducing end of the

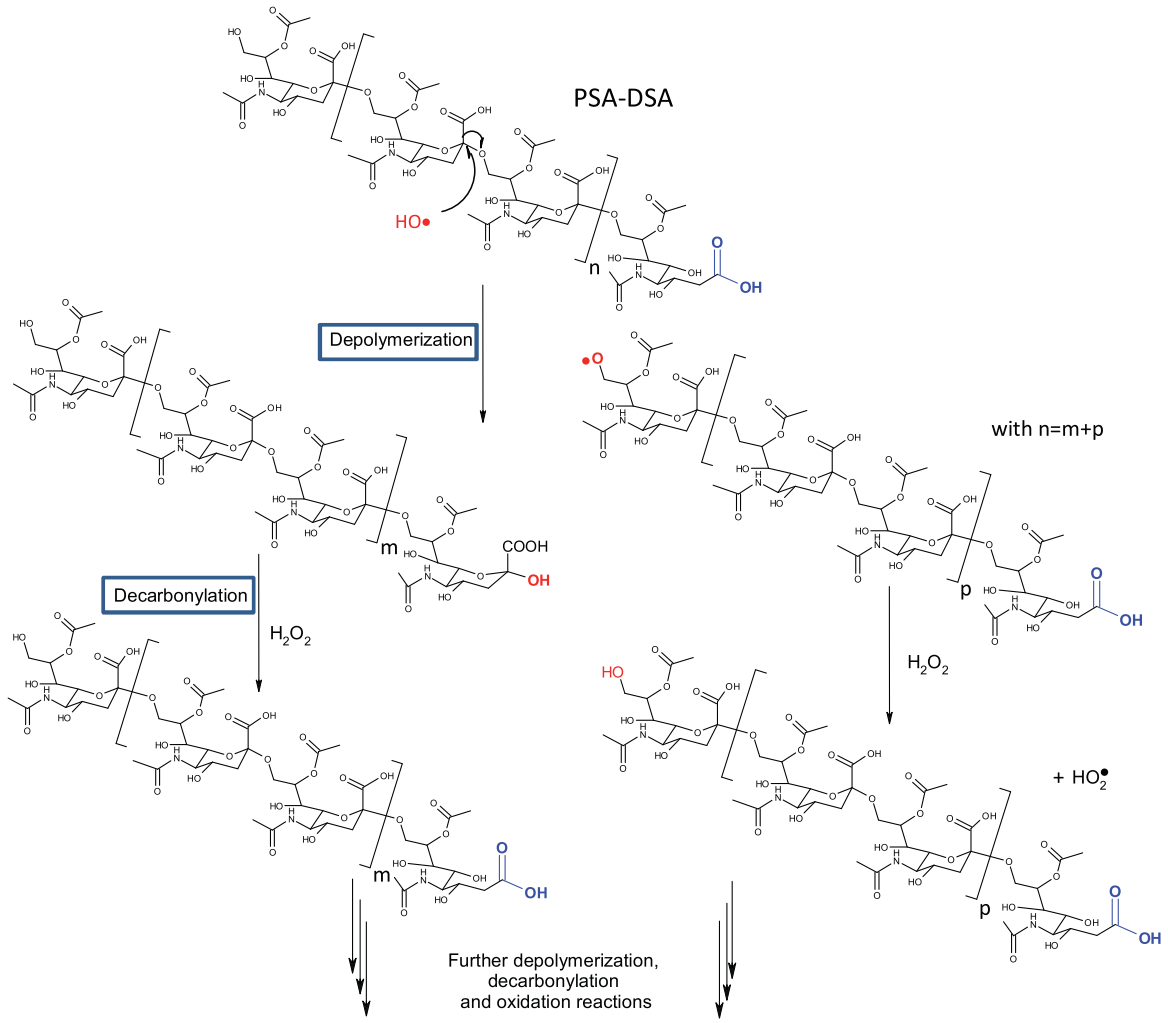


Fig. 8. Cleavage of PSA glycosidic bond by hydroxyl radicals.

depolymerized PSA undergoes a quick decarbonylation leading to the formation of a DSA end.

4. Conclusion

In summary, α-(2 → 9)-linked PSA depolymerized in the presence of H_2O_2 indicates that the structural integrity of the internal sialic acid repeat units on the depolymerized PS is conserved. Additionally, the data suggest that the terminal ends are subject to decarbonylation and oxidation.

Using tetrasialic acid as a model, provided information to elucidate the complex mechanism of depolymerization and oxidation during the reaction between PSA and H_2O_2. It comprised nucleophilic additions, rearrangements, radical-substitutions, hydroxyl group oxidations, and heterolytic alpha-hydroxy-hydroperoxide-cleavages. The carbonyl groups, involved in further functionalization and introduced during the oxidation reactions were observed at monomer level by LC-MS. We can suppose that at the polysaccharide level, the reaction occurs the same way leading to aldehydes and ketones as well.

Currently, studies are underway to optimize the quantity of carbonyl functions generated along the depolymerized polysaccharide chain. Additionally investigations are ongoing to further understand the influence of other promotors (e.g., UV light) and inhibitors of this radical reaction.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.08.112.

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